Neuron, Vol. 33, 177–184, January 17, 2002, Copyright ©2002 by Cell Press

Novel Genomic cDNA Hybrids Produce Effective RNA Interference in Adult *Drosophila*

Neurotechnique

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Summary

Drosophila melanogaster **has been a premier genetic model system for nearly 100 years, yet lacks a simple method to disrupt gene expression. Here, we show genomic cDNA fusions predicted to form double-stranded RNA (dsRNA) following splicing, effectively silencing expression of target genes in adult transgenic animals. We targeted three *Drosophila* genes: *lush*, *white,* and *dGqα*. In each case, target gene expression is dramatically reduced, and the *white* RNAi phenotype is indistinguishable from a deletion mutant. This technique efficiently targets genes expressed in neurons, a tissue refractory to RNAi in *C. elegans*. These results demonstrate a simple strategy to knock out gene function in specific cells in living adult *Drosophila* that can be applied to define the biological function of hundreds of orphan genes and open reading frames.**

Introduction

Manipulating the activity of genes allows the biological role of their protein products to be elucidated. Classical genetic screens combine random mutagenesis with a phenotypic selection to identify genes important for biological processes. However, this approach is laborious and may not identify all relevant genes. Targeted gene knockouts have recently been described in *Drosophila* (Rong and Golic, 2001), but this approach is laborious and the successful use of this approach has not been widely reported in the literature. Furthermore, neither of these genetic methods allows for tissue-specific manipulation of gene expression, which would allow the function of genes in specific cells to be explored. One approach to target gene expression that could circumvent these difficulties is to use double-stranded RNA interference.

Double-stranded RNA interference (RNAi) can reduce gene expression in a sequence-specific manner in diverse organisms by targeting homologous mRNA for degradation (reviewed in Hammond et al., 2001). RNAi can suppress the expression of target genes in *Drosophila* tissue culture cells, *C. elegans*, plants, and in mammalian tissue culture cells (Clemens et al., 2000; Elbashir et al., 2001; Fire et al., 1998; Furner et al., 1998). In *C. elegans*, RNAi is systemic; worms that are fed or injected with double-stranded RNA silence homologous genes throughout the animal and even silence into the next generation (Fire et al., 1998). The power of RNAi in worms has been applied to elucidate the role of genes with unknown functions (Fraser et al., 2000; Gonczy et al., 2000). However, for reasons that are not clear, neurons are resistant to RNAi in *C. elegans* (Timmons et al., 2001).

In *Drosophila*, injection of double-stranded RNA directly into embryos effectively targets homologous gene expression in the embryo (Kennerdell and Carthew, 1998; Misquitta and Patterson, 1999), but effects on adult gene expression are inconsistent and typically modest (Kennerdell and Carthew, 1998). Several groups have recently reported that expression of transgenic "snap-back" inverted repeats (predicted to form dsRNA hairpins) homologous to target genes can reduce gene expression in transgenic flies (Kennerdell and Carthew, 2000; Martinek and Young, 2000; Fortier and Belote, 2000). However, suppression is incomplete and generally produces weak phenotypes. Martinek and Young (2000) quantified the suppression of the *period* gene in transgenic flies and determined there was only a 50% reduction in protein. This level of suppression is insufficient to reliably produce mutant phenotypes for most genes. Additionally, it can be difficult or impossible to recover inverted repeat RNAi constructs in bacteria (Kennerdell and Carthew, 2000; Fortier and Belote, 2000; Piccin et al., 2001). Cloning difficulties led Piccin et al. (2001) to include a GFP spacer between the repeats targeting the *yellow* locus. This facilitated cloning but did not dramatically improve the effectiveness of these constructs compared to uninterrupted repeats. Indeed, *yellow* phenotypes were only observed in flies with three or more transgenic copies regulated by very strong promoters, and the phenotype varied widely between transgenic strains. The low level of genetic suppression described for these RNAi constructs and the difficulties involved in cloning uninterrupted repeat sequences in bacteria led us to explore the effectiveness of RNAi constructs containing introns. Here, we show that genomic cDNA fusions predicted to form hairpin dsRNA molecules following splicing effectively suppress expression of target genes in specific tissues, including neurons. This technique provides a powerful approach to manipulate gene function in specific tissues of adult animals.

Results

Transgenic RNAi to Target LUSH

We used the genomic cDNA RNAi approach to target the *lush* gene (Kim et al., 1998), the *white* gene (Morgan, 1910), and the heterotrimeric G protein α subunit gene, *dGqα* (Lee et al., 1990). Figure 1 shows the design of the RNAi constructs. LUSH is a member of the invertebrate odorant binding protein family. It is expressed at high levels in a small subset of antennal cells (Kim et al., 1998). We fused genomic DNA containing the *lush* promoter and the first three exons and introns to an inverted cDNA fragment encoding exons one, two, and three (Figure 1A). This inverted cDNA region contains no

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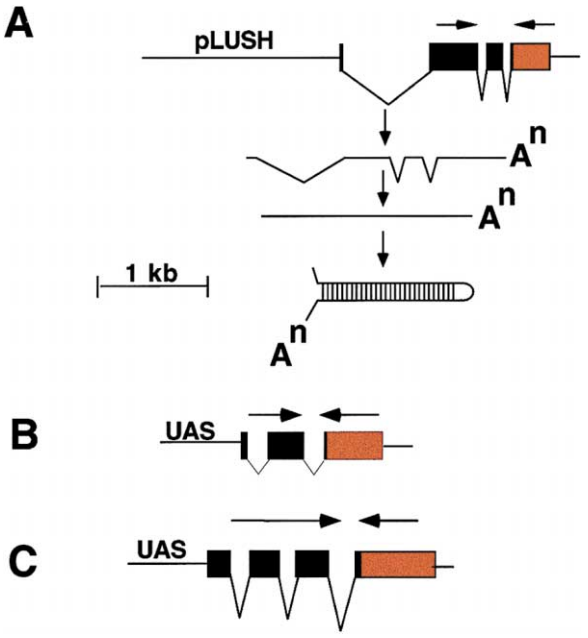

Figure 1. The Structure of the Genomic cDNA RNAi Constructs

(A) *lush* genomic cDNA RNAi fusion construct regulated by its own promoter. 1.6 kb of upstream regulatory sequence, first three exons and introns and splice acceptor of the fourth exon were fused to an inverted *lush* cDNA corresponding to the exons one through three. Transcription of this construct is predicted to first produce the unspliced immature mRNA, which splices to the mature mRNA predicted to form the hairpin double-stranded RNA in the cytoplasm.

(B) Genomic cDNA RNAi construct to target the *white* gene. The second and third exons and introns and splice acceptor of the fourth intron of the *white* locus were fused to an inverted *white* cDNA fragment and cloned into pUAST (Brand and Perrimon, 1993). UAS, *U*pstream *A*ctivation *S*equence. Arrows indicate direction of coding sequence.

(C) Genomic cDNA RNAi construct to target *dGq*α. Genomic DNA including exons three, four, and five (Talluri and Smith, 1995) and the splice acceptor of exon 6A were fused to an inverted *dGq*α-cDNA fragment corresponding to the same region and cloned into pUAST.

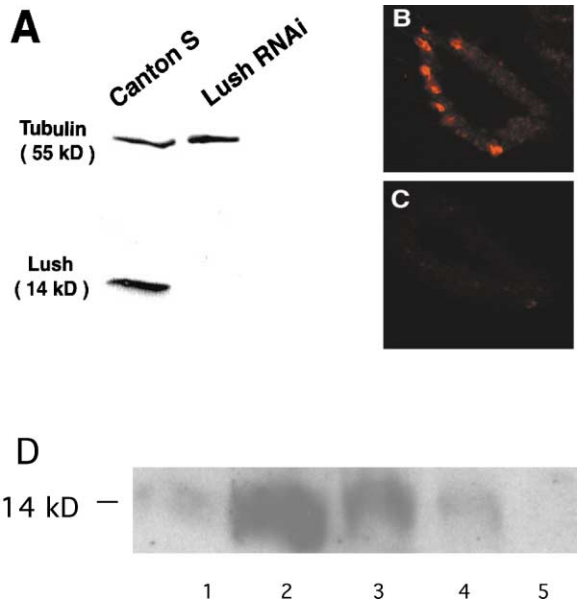

Figure 2. Expression of *lush* RNAi Reduces LUSH Expression

(A) Western blot of antennal extracts from control (Canton S, CS) and transgenic flies expressing the *lush* RNAi construct. Expression of LUSH protein was not detectable, but tubulin levels are equivalent.

(B and C) Immunofluorescent detection of LUSH in frozen tissue sections through the antenna. Control (top panel) and *lush* RNAi transgenic flies (bottom panel). LUSH is readily detectable in the control antenna, but not in the *lush* RNAi-expressing flies. Identical imaging settings were used for both images, and tissues were processed on the same slide.

(D) Western immunoblot of antennal extracts from lush RNAi expressing and control (CS). Lane 1: 50 antennae of Lush RNAi flies. Lane 2: 50 antennae of CS flies. Lane 3: five antennae of CS flies. Lane 4: one antenna of CS, and lane 5: one-half antenna equivalent. 50 Lush RNAi antennae express an equivalent amount of Lush protein found in one-half to one wild-type (CS) antenna.

/GTRAGT sequences that function as splice donors (Mount et al., 1992) that could result in the loss of cDNA sequences in the mature transcript. The use of the *lush* promoter in these constructs ensures the *lush* RNAi will be expressed in the cells that normally synthesize this odorant binding protein (Kim et al., 1998). In contrast to cloning uninterrupted inverted repeats, the genomic cDNA fusions were recovered at high frequency. Figure 2A shows a Western blot containing antennal extracts from 20 wild-type flies and transgenic animals homozygous for the *lush* RNAi construct. The 14 kDa LUSH protein is dramatically reduced in the antennae of flies expressing the *lush* RNAi transgene. The loss of LUSH in the RNAi-expressing flies was confirmed by immunohistochemical staining of frozen tissue sections with anti-LUSH antiserum (Figure 2B). As expected, the LUSH protein was present in the control antennae but was absent from the *lush* RNAi-expressing samples. A faint signal was detectable in the LUSH RNAi antennae at the highest laser intensities (data not shown) but was localized to the cell bodies, and not the sensillum lymph, where this protein is normally concentrated (Kim et al., 1998). Two independent transgenic lines gave indistinguishable results (data not shown). LUSH is first expressed at the late pupal stage, and the LUSH protein is nearly absent from newly eclosed LUSH RNAi-expressing flies. This suggests that *lush* RNAi acts relatively rapidly to suppress expression. From these data, we conclude that transgenic flies expressing the *lush* genomic cDNA RNAi construct have greatly reduced LUSH protein expression.

To quantitate the suppression of LUSH protein in the RNAi-expressing flies, we ran extracts from 50 LUSH RNAi-expressing antennae on a Western blot with serial dilutions of wild-type antenna extracts. Figure 2D shows that 50 LUSH-RNAi antennae express an equivalent amount of LUSH protein found in one-half to one wild-type antenna. Therefore, we conclude that a single homozygous transgenic LUSH RNAi construct suppresses LUSH protein expression between 50- to 100-fold.

Transgenic RNAi to Target the *white* Gene

white encodes an ABC transporter required to localize pigments in eye pigment granules (Mackenzie et al., 2000). Hypomorphic mutations in *white* produce eye

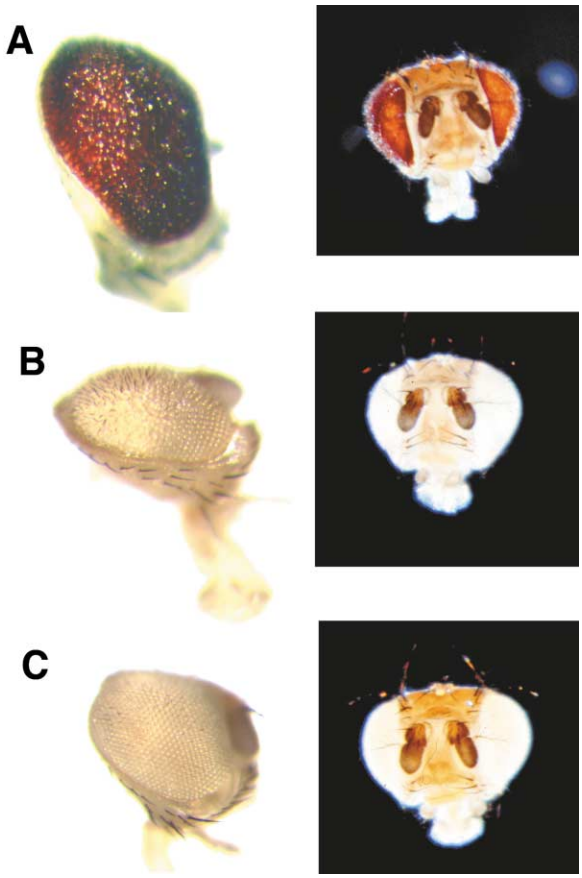

Figure 3. Expression of Genomic cDNA RNAi Construct to the *white* Locus Eliminates Eye Pigmentation

(A) Flies carrying two copies of GMR-Gal4 have darkly pigmented eyes due to expression of two copies of the *miniwhite* gene in the GMR-Gal4 transgene.

(B) Flies carrying two copies of GMR-Gal4 and two copies of UAS *white* RNAi completely lack eye pigmentation despite carrying four total copies of *miniwhite*.

(C) w^{1118} flies are null mutants for white (Flybase, 1999) and are indistinguishable from the white RNAi flies.

colors in various shades of orange, while null mutants have unpigmented white eyes. *white* is a very sensitive indicator of gene expression. Subtle changes in the level of *white* gene expression can be monitored by changes in eye color. We made a genomic cDNA fusion RNAi gene encoding a portion of the *white* locus corresponding to exons two and three. To determine if an open reading frame beginning with an AUG translation intiatiation is important for the stability or effectivness of RNAi suppression, we made constructs with and without a translation initiation site (see supplemental data online at http://www.neuron.org/33/2/177/DC1). We cloned these constructs into the *Drosophila* transformation vector pUAST (Brand and Perrimon, 1993). This vector carries the Gal4 UAS regulatory sequences. By expressing the Gal4 gene under control of a *Drosophila* promoter, we can target the expression of UAS *white* RNAi to cells or tissues of interest. We crossed UAS *white* RNAi flies with and without the translation initiation sequence to a transgenic line expressing Gal4 in the eye, GMR-Gal4 (Hay et al., 1994). Figure 3A shows that GMR-Gal4 flies have dark red eyes. By contrast, GMR-Gal4 flies expressing two copies of the *white* RNAi with the AUG lack eye pigmentation (Figure 3B). Notably, the eye color of these RNAi flies is indistinguishable from that of flies carrying *white* null mutations (Figure 3C). Identical results were obtained with the construct lacking the translation intiation site. Flies expressing a single white RNAi copy had a very faint orange color (data not shown). These results indicate that RNAi constructs composed of genomic cDNA fusions can mimic null mutant alleles and that the potential for the RNAi construct to be translated is probably not important for suppression.

Transgenic RNAi to Target the G Protein α Subunit Gene *dGqα*

As a third target, we chose to direct RNAi against the G protein α subunit gene *dGqα*. This gene is spliced into several mRNAs that encode slightly different G protein α subunits (Talluri and Smith, 1995). One of the splice forms, *dGqα-1*, mediates phototransduction (Scott et al., 1995), while a second splice variant, *dGqα-3*, is widely distributed, but is expressed in a subset of olfactory neurons where it may mediate odor responses (Talluri and Smith, 1995). By selective targeting of a *dGqα*-RNAi construct to photoreceptors or olfactory neurons, we can evaluate the functional impact of RNAi expression and the effectiveness of our constructs in neurons.

The first three coding exons are shared by all splicing variants of *dGqα*. Thus, we used this region of the gene to generate the *dGqα* RNAi, expecting all splicing variants to be targeted (Figure 1C). The *dGqα* RNAi was cloned into pUAST and transgenic flies were generated. To explore the effectiveness of this RNAi construct, we crossed the UAS-*dGqα*-RNAi flies to flies expressing Gal4 under control of the major opsin gene promoter, pRh1. Rh1 opsin is expressed in the outer photoreceptors in each ommatidium or unit eye (Zuker et al., 1985). If the *dGqα* RNAi was effective in these cells, we expected it to phenocopy *dGqα* mutants and greatly reduce the sensitivity of these cells to light. We examined the transgenic flies expressing dGqα in the photoreceptors and confirmed that they were wild-type for expression of control markers including tubulin and rhodopsin (Figure 4A).

Antiserum specific to dGqα-1, the splice variant that mediates vision, detects protein in control flies (Oregon R, lane 1), but not in transgenic flies expressing *dGqα* RNAi in the R1-R6 photoreceptors (Figure 4A, lane 3). Indeed, *dGqα-1* is almost completely lacking in the transgenic flies. Identical results were obtained for a second independent transgene line. Residual dGqα-1 expression apparent upon prolonged exposures (Figure 4B) may represent protein expressed in R7 and R8 photoreceptors that do not express Rh1 opsin or may result from incomplete suppression of *dGqα* in R1-R6 cells by a single RNAi transgene. Figure 4C demonstrates that flies expressing one copy of *dGqα* RNAi in their photoreceptors have a 1000-fold reduction in sensitivity to light. This is similar to what is seen on extracellular recordings of strong hypomorphic *dGqα-1* mutants (Scott et al., 1995). We conclude that expressing a single copy of the *dGqα*- RNAi construct in photoreceptor neurons effectively reduces the levels of functional dGqα protein in the photoreceptor neurons of these living adult animals.

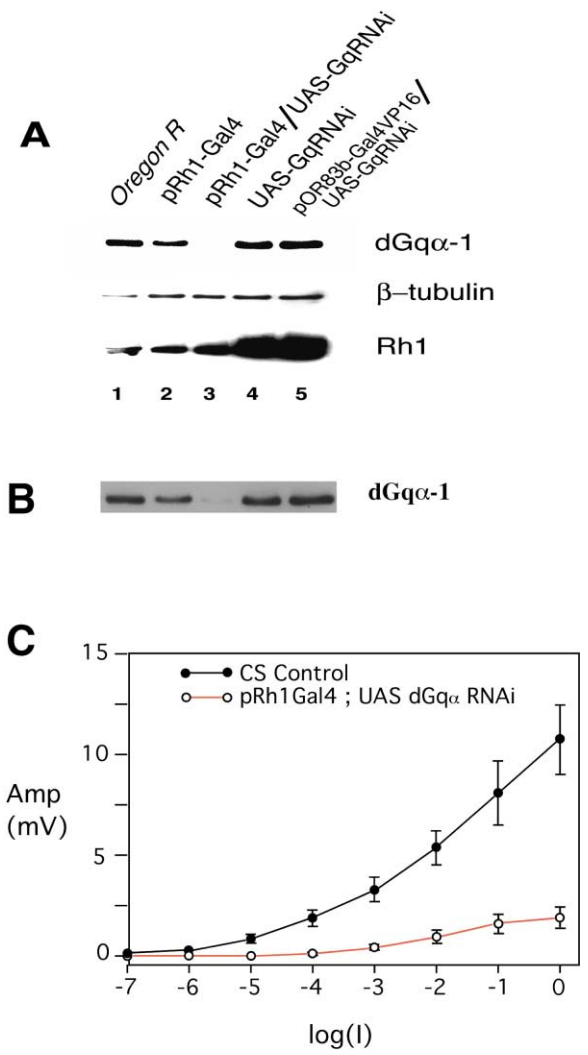

Figure 4. Expression of Genomic cDNA RNAi Construct to *dGqα* in the R1-R6 Photoreceptor Cells

(A)Western blot showing the loss of dGqα expression in transgenic flies expressing a single copy of *dGqα* RNAi under control of the Rh1 promoter. Lane 1: Oregon R control (wild-type). Lane 2: pRH1-Gal4 transgene alone. Lane 3: pRh1-Gal4, UAS *dGqα* RNAi. Lane 4: UAS *dGqα* RNAi alone. Lane 5: pOR83b-Gal4VP16, UAS *dGqα* RNAi. Expression is not affected by either transgene alone, eliminating potential effects of the P element integrations on dGqα expression (lanes 2 and 4), but potent RNAi is observed when a single copy of each transgene is present in the same flies (lane 3). Expression of control proteins (tubulin and rhodopsin) are not significantly different among the lanes showing that the R1-R6 photoreceptors are still present, have not changed cell fate and still express other photoreceptor-specific proteins. Expression of *dGqα* RNAi in antennal cells using the promoter for OR83b (lane 5) does not affect dGqα expression in the eye, demonstrating that transgenic RNAi is not systemic in *Drosophila*.

(B) Western blot of dGqα on prolonged exposure. Residual dGqα expression apparent may represent protein expressed in R7 and R8 photoreceptors or incomplete suppression of the same in R1-R6 cells by a single transgene.

(C) Comparison of the amplitude of the light-induced electrical responses from control and flies expressing *dGqα* RNAi under control of the Rh1 promoter. Extracellular recordings (ERGs) were performed in response to 480 nm light flashes at a range of light intensities. A single dose of the RNAi construct reduces the amplitude of the response. Sensitivity is reduced by 1000-fold. Error bars represent standard error of measurement. Responses were averaged for seven wild-type and six RNAi flies for each light intensity.

A Role for Gq Signaling in Olfactory Neurons

Next, we used the *dGqα*-RNAi construct to explore the role of *dGqα* signaling in olfactory transduction. The biochemical mechanisms that mediate chemosensory signaling in insects are unknown, but the identification of seven transmembrane receptors expressed in most olfactory neurons strongly argues for a G protein signaling mechanism. dGqα-3 is a candidate for transducing olfactory signaling in olfactory receptor neurons. However, dGqα-3 is widely expressed in adults. Thus, to explore its role in olfaction, we targeted expression to a subset of olfactory neurons. We cloned the upstream regulatory sequences of the OR83b receptor, a putative odor receptor expressed in a large fraction of olfactory neurons (Vosshall et al., 1999). We created flies that express Gal4-VP16 under control of OR83b regulatory sequences and crossed them to flies expressing a myc-tagged version of the OR83b receptor under UAS control. OR83b-myc reporter expression is specifically restricted to most, but not all, olfactory neurons (Figure 5A). No transgene expression was detected outside the olfactory organs.

Figures 5B and 5C show that flies expressing *dGqα*−RNAi constructs in the olfactory neurons with the OR83b promoter produce odor-specific defects in olfactory behavior. For example, the behavioral responses to isoamyl acetate are dramatically altered using T maze olfactory behavior assays (Tully and Quinn, 1985). At isoamyl acetate concentrations ranging from 10^{-5} to 10^{-2} M, flies expressing *dGqα* RNAi in olfactory neurons appear insensitive to this compound compared to controls (Figure 5B). Responses to benzaldehyde are altered in a more complex manner (Figure 5C). At 10^{-5} M, there is little difference among the groups to benzaldehyde. However, at 10^{-4} M, benzaldehyde induces avoidance behavior in RNAi-expressing flies, but still elicits attraction in controls. All genotypes are equally repelled by benzaldehyde at 10^{-3} and 10^{-2} M. Responses to diluent (paraffin oil), crushed apples, or *Drosophila* food are not different between the two groups (Figure 5D). Therefore, behavioral responses to a subset of odorants are altered by expression of a *dGqα*-RNAi construct in olfactory neurons, but responses to other odorants are not affected.

To determine if expression of *dGqα* RNAi in olfactory neurons affects olfactory behavior in a test mimicking chemotactic behavior in the wild, we assayed the ability of *dGqα*-RNAi flies to chemotax to small traps containing an attractant located within a large population cage. Figure 5E shows that in contrast to control flies, the *dGqα*-RNAi-expressing flies are impaired in the ability to chemotax into traps containing yeast extract.

In plants, RNAi is non-cell-autonomous; grafting plant components expressing RNAi transgenes results in suppression throughout the plant (Palauqui et al., 1997). Expression of the *dGqα*-RNAi construct in olfactory neurons does not reduce expression of dGqα in the eye, indicating the RNAi effect does not spread throughout

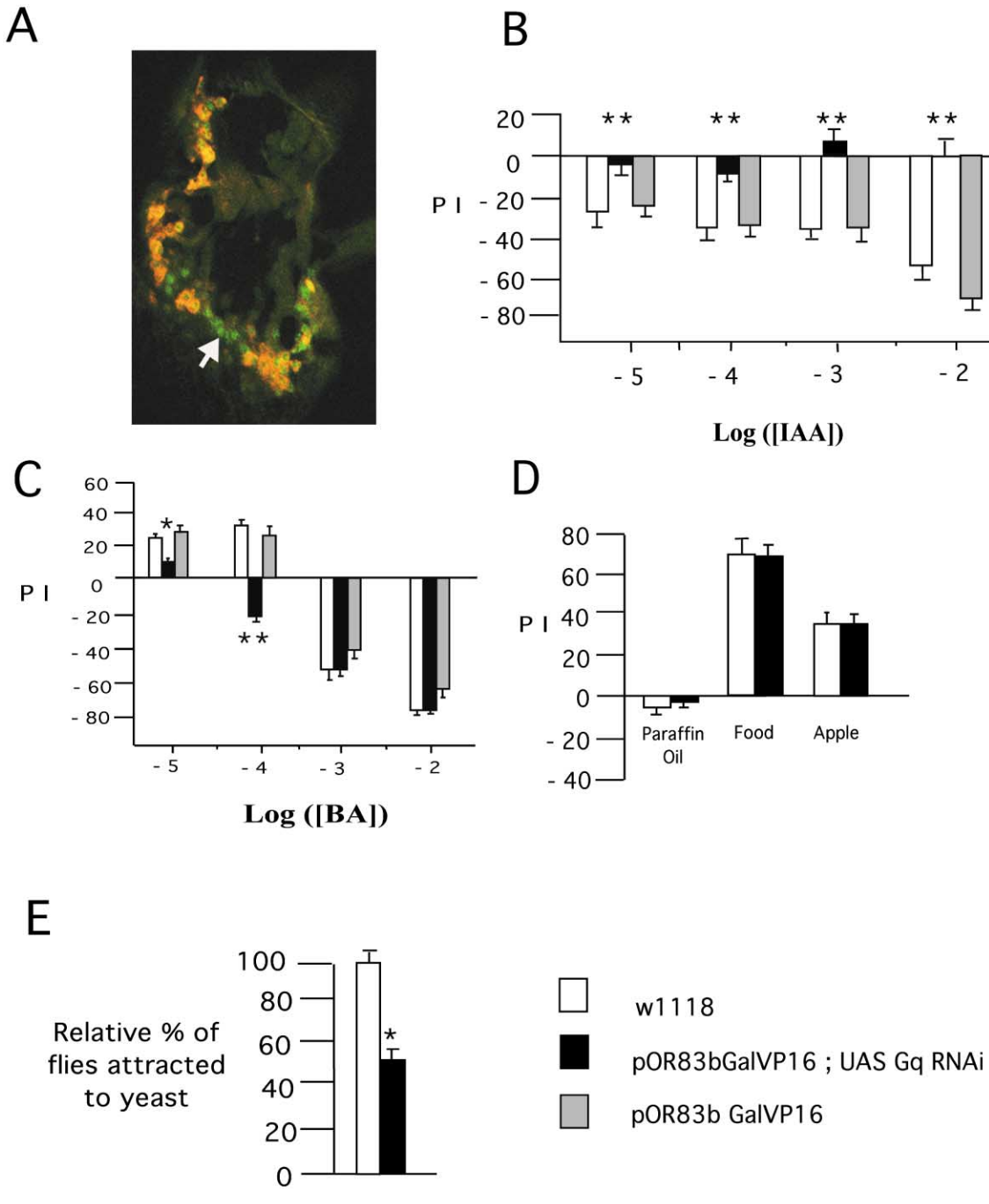

Figure 5. Expression of *dGqα* in Olfactory Neurons Disrupts Olfactory Behavior

(A) Promoter for OR83b drives Gal4-VP16 expression in a subset of olfactory neurons. ELAV antibodies label all neuronal nuclei in the antenna (green). In this antennal section, all neurons are expected to be primary olfactory neurons. OR83b promoter drives Gal4-VP16 expression in a subset of olfactory neurons detected with the reporter transgene UAS-OR83b-myc (red). All myc-positive cells are neurons. A subset of neurons are present that express ELAV, but not myc (arrow), indicating the OR83b promoter drives expression in a subset of olfactory neurons.

(B–D) Olfactory behavior of transgenic flies expressing *dGqα* RNAi in olfactory neurons is abnormal. Positive PI indicates attraction, negative PI avoidance, and PI of 0 indicates neither attracted nor repulsed. (B) Control flies (white and gray bars) avoid isoamyl acetate, but *dGqα* RNAi flies (black bars) appear insensitive to this odorant.

(C) Responses to benzaldehyde are also abnormal when *dGqα* RNAi is expressed in olfactory neurons, but the responses to apple and food (D) are not affected. Expression of OR83b Gal4-VP16 alone does not affect the olfactory behavior (gray bars). Bars represent standard error of measurement. Significance was determined using the Student's t test.

(E) Open chamber chemotaxis to yeast is reduced in flies expressing *dGqα* RNAi in olfactory neurons.

An asterisk indicates significantly different from controls at $p \leq 0.05$; a double asterisk indicates significantly different from controls at $p \leq 0.005$.

the animal (Figure 4A, lane 5). This data suggests that the RNAi effect is not systemic and may be cell-autonomous in *Drosophila* when expressed as transgenes.

Discussion

We show that RNAi transgenes composed of genomic cDNA fusions efficiently reduce gene expression of target genes in adult flies. We demonstrate this at the protein level (LUSH and dGqα), at the gross phenotypic level (*white*), at the physiological level (*dGq*α), and the behavioral level (*dGq*α). The level of suppression we observe is significantly greater and more uniform between transgenic lines than previous reports using transgenic RNAi constructs composed of simple inverted repeats (Kennerdell and Carthew, 2000; Martinek and Young, 2000; Fortier and Belote, 2000) or inverted repeats separated with a spacer (Piccin et al., 2001). Kennerdell and Carthew (2000) showed that inverted repeat snap-back RNAi constructs expressed as transgenes could suppress LacZ expression in embryos, but the effects were variable and incomplete. Heat shock-expressed LacZ RNAi expressed in older animals produced modest reductions in β-galactosidase activity. Fortier and Belote (2000) described an inverted repeat construct to *tra-2* that produced weak sex-transforming phenotypes. They described difficulties in cloning the constructs, and some were never obtained. The best results achieved were with four transgenic copies of the *tra-2* RNAi construct driven by the potent actin promoter. Even so, they were unable to completely phenocopy the *tra-2* external genitalia phenotype. They also noted variability among transgenic lines. Martinek and Young (2000) used snap-back transgenic constructs to target the *period* gene and demonstrated modest alterations in circadian rhythm in these transgenic flies. They went on to quantitate *per* RNA and demonstrated a 50% reduction in transcripts. This level of reduction is unlikely to produce a phenotype in genes that are less sensitive to dosage. Finally, Piccin et al. (2001) used a spacer between inverted repeats to facilitate cloning of RNAi constructs to target *yellow*. Multiple copies of the RNAi constructs (three to six copies) were required to produce strong phenotypes, even when expressed under actin or heat-shock promoters. Therefore, including a spacer between the repeats facilitates cloning but does not appear to dramatically improve the efficiency of suppression. These workers also noted variability among transgenic lines. These difficulties led us to try transgenic RNAi constructs containing introns. While we have not directly compared the effects of inverted repeat constructs to those containing introns using the same gene, the efficacy of our constructs at even single copies represents a great improvement over these previous studies and suggests that splicing may be important to efficiently produce RNAi with transgenes. For example, the yeast splicing factor SUB2 interacts directly with YRA1, an essential component of the mRNA export machinery, suggesting that splicing and mRNA export are coupled (Straber and Hurt, 2001). One possibility is that spliced RNAi transcripts are more efficiently processed and therefore reach higher levels in the cytoplasm. This mechanism could account for the potency of our constructs relative to RNAi constructs lacking introns. This notion is supported by the finding that an artificial intron placed between inverted repeats induces greater supression in plants (Smith et al., 2000). Alternatively, transcripts encoding inverted repeats may be unstable owing to the propensity of these molecules to form hairpins during transcription, and including introns may reduce hairpin formation in premRNAs.

In contrast to plants and *C. elegans*, we show transgenic RNAi in *Drosophila* does not affect expression of the same gene in other tissues, demonstrating transgenic RNAi is not systemic in *Drosophila*. This suggests that transgenic RNAi constructs may be cell-autonomous in flies. However, we have not ruled out the possibility that RNAi silencing spreads locally. A conclusive demonstration of cell autonomy could be done by generating mitotic clones expressing RNAi and assaying for suppression in neighboring cells. However, the fact that RNAi silencing is not systemic should allow for analysis of gene function in specific tissues, even with genes that are lethal when globally absent. We show genomic cDNA RNAi is capable of targeting genes expressed in diverse tissues, including neurons, making this a useful tool to elucidate the role of any *Drosophila* gene.

Finally, we have applied this technique to explore the role of dGqα in olfactory behavior. Null mutations in this gene have not been recovered, probably because these mutants are lethal (D.P.S., unpublished data). Furthermore, in addition to neurons, *dGq*α is expressed in nonneuronal tissues in the antenna where its absence could indirectly affect olfactory behavioral responses (Talluri and Smith, 1995). Therefore, targeting *dGq*α RNAi in olfactory neurons is one of the few ways to determine if this protein is required in these cells for normal olfactory behavior. When *dGq*α RNAi is expressed in a large subset of olfactory neurons, responses to isoamyl acetate are abolished, indicating behavioral reponses to this odorant require Gqα in these olfactory neurons. Responses to benzaldehyde are altered in a more complex way. We observe a concentration-dependent shift in the behavioral responses to this odorant. At 10^{-5}, benzaldehyde is slightly less attractive to the RNAi-expressing flies compared to controls. At 10^{-4}, BZ acts as repellant to the RNAi flies, while still attracting controls. These data are consistent with a selective defect in attraction to benzaldehyde. Avoidance of benzaldehyde, as well as responses to odors not affected by the RNAi construct, may be mediated through alternate signaling mechanisms, perhaps through different G proteins. Alternatively, olfactory behavioral responses not affected by our RNAi construct may be mediated by the subset of olfactory neurons not expressing the construct. These results provide direct in vivo evidence that heterotrimeric G proteins of the Gq mediate olfaction in insects.

This RNAi approach provides a powerful tool to effectively manipulate gene expression in flies that will facilitate the task of correlating specific genes with biological functions. The availablility of a vast array of Gal4 drivers capable of expressing Gal4 in any number of temporal or spatial expression patterns should allow for the widespread applicability of this approach. The recent finding that *let-7*, a small noncoding RNA encoding an endogenous RNAi hairpin likely to function by forming dsRNA, has a conserved vertebrate homolog (Pasquinelli et al.,

2000), indicates that modification of our approach may find application in vertebrate systems in the near future.

Experimental Procedures

Drosophila Stocks and Transgenic Flies

Oregon R, Canton S, and w^{1118} were used as controls in these studies. Rh1-Gal4 flies were provided by Rama Ranganthan. Transgenic flies were generated as previously described (Karess and Rubin, 1984). GMR-Gal4 was described in Hay et al. (1994). Transgenic RNAi flies were homozygous for the transgenes with the exception of the pRh1 Gal4; UAS *dGq*α RNAi flies that carry a single copy of each transgene. In all cases, a minimum of two independent-transformant strains were compared for each construct.

RNAi Constructs

Genomic and cDNA fragments were amplified using PCR with primers containing unique restriction sites. See supplemental data (online at http://www.neuron.org/33/2/177/DC1) for specific construct information and for a protocol to facilitate generation of RNAi constructs. PCR products were cloned using TA cloning kit (InVitrogen, Carlsbad CA) and sequenced. Fragments were selected to avoid splice donor sequences (/GTNNGT) within the inverted cDNA (Mount et al., 1992). To disrupt direct repeats as much as possible, cDNA sequences were fused to the genomic sequence just after a splice acceptor. For *lush*, the genomic PCR included 1.6 kb of regulatory sequence upstream of the first noncoding exon and contained a Not1 site at the 5′ end and a Asc1 site at the 3′ end. This was ligated to a *lush* cDNA fragment corresponding to the genomic region that was isolated using PCR primers containing an Asc1 site at the 5′ end and an Xba site at the 3′ end. These fragments were cloned into Bluescript *lush* A^n (containing the 3′ untranslated portion of *lush* as a EcoR1-Sal1 fragment) as a Not1 to Xba1 fragment. The completed construct was digested with Not1 and Sal1 and cloned into Casper4 (Pirrotta, 1988) digested with Not1 and Xho1.

For *white*, the RNAi construct was targeted to the second and third coding exons. The genomic portion was isolated by PCR as an EcoR1 to Not1 fragment and ligated to the inverted cDNA sequence as an Not1 to Xho1 fragment in pUAST digested with EcoR1 and Xho1.

For the *dGq*α-RNAi construct, the genomic fragment was isolated by PCR as an EcoR1 to Not1 fragment and ligated to the inverted cDNA sequence digested with Not1 to Xho1 and cloned into pUAST digested with EcoR1 and Xho1. Ligated RNAi constructs were transformed into SURE2 cells (Stratagene, LaJolla CA).

Primers sequences for RNAi constructs were:
*DGq*α genomic forward, GGGAATTCAAAATGGAGTGCTGTTTAT CGGAGGAG; *DGq*α genomic reverse, GGGCGGCCGCCTGTACATA GACAAGATAATTGAT; *DGq*α cDNA forward, GGGCGGCCGCTAT TTGGCTGAATCAGTCAGCTG; *DGq*α cDNA reverse, GGCTCGAG AAAATGGAGTGCTGTTTATCGGAGGAG; *white* genomic forward, GGGAATTCAAAATGGGCTACCGGCGCCCAGGAAAC; *white* genomic reverse, GGGCGGCCGCCCACGCTGGATAGGAGTTGAGATGT; *white* cDNA forward, GGGCGGCCGCTAGGAAAAGAAGTCGACGG CTTCGC; *white* cDNA reverse, GGCTCGAGATGGGCTACCGGCGC CCAGGAAACATT; *lush* genomic forward, GGGCGGCCGCTCAAT TAACCCTCACTAAAG; *lush* genomic reverse, GGGGCGCGCCTTTA TCTGCAAAGATATACC; *lush* cDNA forward, GGGGCGCGCCTTTA TGCGTATCCCGACAAG; *lush* cDNA reverse, GGGTCTAGAATGAA GCATTGGAAACGACGC.

The OR83b promoter was cloned from genomic DNA using PCR and included Pac1 and Asc1 sites on the primers to facilitate directional cloning. 8 kb of DNA immediately upstream of the predicted starting Methionine was cloned into pGal4-VP16 (Galindo and Smith, 2001) as a Pac1 to Asc1 fragment. PCR primers to isolate the OR83b promoter were:

Forward, GGTTAATTAATGGTGTCCTGCAGCGACAACAGTCTGC TCC; reverse, GGGGCGCGCCCATCCTGTTGAGCGGCGGAAATTG CACGAC.

Western Blotting and Immunofluorescence

LUSH was detected with affinity-purified anti-LUSH rabbit antiserum described previously (Kim et al., 1998). Control and *lush* RNAi sections were processed on the same slides to insure identical exposure to reagents.

Drosophila Behavioral Assays

T tube odor choice tests were performed as previously described (Tully and Quinn, 1985). Performance index (PI) was calculated as ([number of flies attracted to odor/total number flies] $\times$ 2 $-$ 1) $\times$ 100.

Open chamber experiments were performed in 24 $\times$ 24 in population cages containing 100 flies of each genotype. Traps consisted of borosilicate test tubes containing 5% yeast extract in 1% agarose. Flies were allowed to interact with the traps for 8 hr, and the flies within the traps were sorted by eye color and counted. The experiment was repeated three times and statistical analysis was performed using the Student's t test.

Electrophysiology

Electroantennograms recordings (ERG) were performed on flies mounted on the end of a 200 μl ependorf tip and immobilized with myristic acid. Glass electrodes were filled with standard saline (0.7% NaCl). Light stimuli were delivered by a 100 W Xenon arc lamp (Oriel, Stratford, CT) using an interference filter (480 $\pm$ 10 nm); (Oriel, Stratford, CT). Neutral density filters (Oriel, Stratford, CT) were used to produce different light intensities. All recordings were performed under dim red light. Flies were stimulated by light pulses of 2 s duration, and responses were amplified using a differential amplifier (Warner Instruments, Handen, CT), filtered at 1 kHz, and digitized at 300 kHz. Data were collected using Clampex V8.0 (Axon Instruments, Foster City, CA) and analyzed using Matlab 6.1 (Mathworks Inc., Natick, MA). Flies were allowed to recover for 1 min between flashes.

Acknowledgments

The authors thank Tamara Elmore for helping to characterize the OR83b promoter; Mike Socolitch and Rama Ranganathan for assistance with the ERG studies; and Al Gilman, Charles Zuker, Tom Südhof, and members of the Smith lab for reviewing the manuscript. This work was supported by NIH DC02539 and by the American Heart Association, EI0040029N.

Received October 19, 2001; revised December 6, 2001.

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